Synergistic action of estradiol and PGE2 on endometrial transcriptome in vivo resembles pregnancy effects better than estradiol alone

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Abstract: Successful pregnancy establishment in mammals depends on numerous interactions between embryos and the maternal organism. Estradiol-17 (E2) is the primary embryonic signal in the pig, and its importance has been questioned recently. However, E2 is not the only molecule of embryonic origin. In pigs, prostaglandin E2 (PGE2) is abundantly synthesized and secreted by conceptuses and endometrium. The present study aimed to determine the role of PGE2 and its simultaneous action with E2 in changes in porcine endometrial transcriptome during pregnancy establishment. The effects of PGE2 and PGE2 acting with E2 were studied using an in vivo model of intrauterine hormone infusions, and were compared to the effects of E2 alone and conceptuses' presence on day 12 of pregnancy. The endometrial transcriptome was profiled using gene expression microarrays followed by statistical analyses. Downstream analyses were performed using bioinformatics tools. Differential expression of selected genes was verified by quantitative PCR. Microarray analysis revealed 2413 differentially expressed genes (DEGs) in the endometrium treated simultaneously with PGE2 and E2 ($P < 0.01$). No significant effect of PGE2 administered alone on endometrial transcriptome was detected. Gene ontology annotations enriched for DEGs were related to multiple processes such as: focal adhesion, vascularization, cell migration and proliferation, glucose metabolism, tissue remodeling, and activation of immune response. Simultaneous administration of E2 and PGE2 induced more changes within endometrial transcriptome characteristic to pregnancy than infusion of E2 alone. The present findings suggest that synergistic action of estradiol-17 and PGE2 resembles the effects of pregnancy on endometrial transcriptome better than E2 alone.

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Title: Synergistic action of estradiol and PGE2 on endometrial transcriptome in vivo resembles pregnancy effects better than estradiol alone.

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Summary sentence: Prostaglandin E2 acting simultaneously with estradiol-17β in vivo induces more changes within porcine endometrial transcriptome resembling pregnancy effects compared with estradiol-17β administered alone.

Keywords: early pregnancy; endometrium; pig; prostaglandins; estradiol-17β; transcriptome profiling

Additional footnotes: The presented data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus and are accessible through GEO Series accession number GSE158568.

ABSTRACT

Successful pregnancy establishment in mammals depends on numerous interactions between embryos and the maternal organism. Estradiol-17β (E2) is the primary embryonic signal in the pig, and its importance has been questioned recently. However, E2 is not the only molecule of embryonic origin. In pigs, prostaglandin E2 (PGE2) is abundantly synthesized and secreted by conceptuses and endometrium. The present study aimed to determine the role of PGE2 and its simultaneous action with E2 in changes in porcine endometrial transcriptome during pregnancy establishment. The effects of PGE2 and PGE2 acting with E2 were studied using an in vivo model of intrauterine hormone infusions, and were compared to the effects of E2 alone and conceptuses' presence on day 12 of pregnancy. The endometrial transcriptome was profiled using gene expression microarrays followed by statistical analyses. Downstream analyses were performed using bioinformatics tools. Differential expression of selected genes was verified by quantitative PCR. Microarray analysis revealed 2413 differentially expressed genes (DEGs) in the endometrium treated simultaneously with PGE2 and E2 ($p<0.01$). No significant effect of PGE2 administered alone on endometrial transcriptome was detected. Gene ontology annotations enriched for DEGs were related to multiple processes such as: focal adhesion, vascularization, cell migration and proliferation, glucose metabolism, tissue remodeling, and activation of immune response. Simultaneous administration of E2 and PGE2 induced more changes within endometrial transcriptome characteristic to pregnancy than infusion of E2 alone. The present findings suggest that synergistic action of estradiol-17β and PGE2 resembles the effects of pregnancy on endometrial transcriptome better than E2 alone.

1. INTRODUCTION

The peri-implantation period in mammalian species is a critical stage characterized by a high risk of pregnancy establishment failure. During this time developing embryos signal their presence to the maternal organism by secretion of multiple factors including hormones, cytokines, and other molecules (reviewed in [1]). In pigs, the primary embryonic signal is estradiol-17β (E2), which is abundantly synthesized and secreted by conceptuses (embryos with associating membranes) on days 11–13 and 15–25 post estrus [2, 3]. Elevated estrogens synthesis and secretion by porcine conceptuses initiates the maternal recognition of pregnancy involving multiple processes, resulting in a prolonged lifespan of corpus luteum and the maintenance of progesterone secretion [4]. Progesterone, in turn, induces the uterine receptivity that corresponds to the limited period of time during which the uterine luminal epithelium is favorable to blastocyst implantation [5].

During the maternal recognition of pregnancy, the uterine endometrium undergoes rapid morphological and functional changes enabling its readiness for conceptus attachment [6, 7]. These changes occurring in the endometrium during the peri-implantation period are preceded by dynamic alterations within the endometrial transcriptome [8–11]. Intriguingly, recent report based on results from studies involving CYP19A1-deficient porcine embryos have contested the role of E2 in early embryonic development, conceptus elongation, maternal recognition of pregnancy, attachment to the maternal epithelium, placentation, and maintenance of pregnancy until day 30–35 [12]. In contrast, we recently evidenced an important role of estradiol-17β in the induction of changes in the global gene expression profile of the porcine endometrium in vivo that were characteristic of early pregnancy [13].

Embryonic estrogens, however, are not the only molecules involved in the establishment of pregnancy in the pig. Our studies indicated that in pigs, prostaglandin E2

(PGE2) is synthesized and secreted both by conceptuses and the endometrium during the period corresponding to the maternal recognition of pregnancy [14–16]. PGE2 acting as a conceptus signal mediator has been suggested to be involved in prolonging the corpus luteum lifespan during early pregnancy in pigs [17-19]. Moreover, we evidenced that PGE2 acting through its receptors in the endometrium during the peri-implantation period stimulates conceptus PTGER2 expression and increases the synthesis and secretion of estradiol-17β. Additionally, PGE2 enhances integrin-dependent trophoblast adhesion [20]. However, recent studies involving prostaglandin-endoperoxide synthase 2 (PTGS2) knockout embryos implied that prostaglandins of conceptus origin are not essential for early development and establishment of pregnancy in the pig [21].

Our previous studies demonstrate the importance of PGE2 to processes that support conceptus attachment to the endometrium for implantation in pigs. However, recent controversial reports potentially diminish the role(s) of PGE2 and E2 in embryonic development and the establishment of pregnancy. Therefore, we aimed to determine whether PGE2 may be responsible for inducing changes within the endometrial transcriptome and to resolve the synergistic effects of PGE2 and E2 on changes in the global gene expression profile during early pregnancy. In our studies we applied an in vivo model of hormonal infusions administered directly into the uterine lumen, and included endometrial samples collected from gilts on day 12 of pregnancy and the estrous cycle as a reference group. To determine the effects of PGE2 and PGE2 acting simultaneously with E2 in changes occurring in the porcine endometrium, we performed transcriptome profiling using gene expression microarrays followed by downstream functional annotation analyses applying bioinformatics tools.

2. MATERIALS AND METHODS

All procedures involving the use of animals were conducted in accordance with the national guidelines for agricultural animal care and were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland, permission No. 17/2008.

1.1. Animal model in vivo

Effects of PGE2 administered alone or simultaneously with estradiol-17β were studied using a porcine in vivo model. Crossbred gilts at the same age and genetic background, after second natural estrous cycle underwent estrus synchronization according to the procedure described previously [22]. On day 8-9 of the third natural estrous cycle animals underwent surgery as described previously [13, 22]. Briefly, the intrauterine catheter was perforated along its length (30 cm silicone tube with a blind end and perforated every 5 cm) in order to simulate hormone delivery by embryos. The catheters were introduced into the uterine lumen of both uterine horns at a distance of 10 - 15 cm from the isthmus and they did not cover whole uterine horn. The catheters were exteriorized through a small flank incision on their adjacent side. Mixing between uterine horns was excluded. Animals were then divided in four groups (n=5-6 per group). In the control group, each horn received intrauterine placebo (vehicle, 5 mL of 1% v/v ethanol saline) infusions. In hormone-treated groups, randomly selected horns within each gilt received hormonal infusions: PGE2 (200 µg/infusion; n=6), E2 (33.3 µg/infusion; n=5), or PGE2 (200 µg/infusion) simultaneously with E2 (33.3 µg/infusion; n=6). The contralateral horn received placebo infusions. Doses of hormones used were similar to those previously published [23-25]. In our previous report [13] we evidenced that a lower dose of E2 (833 ng/infusion) resulted in alterations within the endometrial transcriptome similar to pregnancy effect. However, a higher dose of E2 (33.3 µg/infusion)

induced greater changes in the global gene expression profile that were more similar to changes identified in the endometrial transcriptome on day 12 of the pregnancy. Treatments were administered manually by using sterile syringes plugged into exteriorized catheter every 4 h for 24 h beginning on the morning of day 11 after the onset of estrus. After experiment, on day 12 since the estrus, gilts were slaughtered at the local abattoir and uteri were collected. Endometrial tissue was dissected from myometrium and snap-frozen in liquid nitrogen, then stored at -80 °C until further analyses.

1.2. Total RNA isolation and synthesis of complementary DNA

Total RNA was isolated from endometrial samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Purity and concentration of isolated RNA was measured with NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed by using Agilent Eukaryote Total RNA Nano chips and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To synthesize the cDNA for quantitative PCR (qPCR) reaction, a 1 µg of total RNA sample was reverse-transcribed by using MultiScribe™ Reverse Transcriptase kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Reverse-transcribed cDNA samples were stored in -80 °C for further qPCR analyses.

1.3. Endometrial transcriptome profiling by using expression microarrays

Total RNA (100 ng) isolated from endometrial tissues was used in transcriptome profiling by using expression microarrays. Cy3-labeled cRNA was generated with the Low-Input Quick Amp Labeling Kit, one-color (Agilent Technologies) and hybridized to the Agilent 4x44k Porcine Gene Expression microarrays (G2519F-026440) according to the

protocol provided by the producer. Following hybridization and washing slides were scanned with an Agilent DNA Microarray Scanner (G2505C; Agilent Technologies) at 2-µm resolution. Feature Extraction Software 10.5.1.1 (Agilent Technologies) was used for image processing. Expression signals were filtered based on "well above background" flags (detection in 5 of 10 samples in control group, and in 5 of 6 samples in experimental group) and normalized with the BioConductor package VSN [26]. For quality control, normalized data were analyzed with a distance matrix and a heatmap based on pair-wise distances (BioConductor package Geneplotter). Probes exhibiting significant differences in signal intensity were identified in the contrasts: "hormone-treated horns" (experimental group) vs. "placebo-treated horns" (control group) using the BioConductor package LIMMA (Linear Model for Microarray Analysis; [27]). Following cut-off's were applied: log fold change > 0.585 and P-value < 0.01. For the correction of multiple testing the "FDR"-method (False Discovery Rate; FDR 5%, i.e., adjusted P-value < 0.05) was applied. The distribution of expression signals of identified probes among samples, was visualized by hierarchical clustering using Pearson correlation performed in Multiexperiment Viewer software (MEV; [28]). Probes were annotated based on mapping of their sequences (60 nt sequences) to the porcine genome (SusScrofa 11.1). Known and potential human ortholog or homolog genes were assigned using a ortholog annotation database (Mammalian Ortholog and Annotation Database, MAdb; [29]). If a gene in the list was represented by more than one probe sequence, the mean of log fold-change, P-value and adjusted P-value (FDR) were calculated. The presented data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus and are accessible through GEO Series accession number GSE158568.

2.4. Functional annotation of microarray results

Lists of differentially expressed genes (DEGs) generated by LIMMA analysis was used as input data for functional annotation. To study the changes in global gene expression profile in endometrial samples treated with PGE2 and E2, we used open-source and commercial software. The Database for Annotation, Visualization, and Integrated Discovery (DAVID; [30; 31]) was used to calculate the fold enrichment of identified gene ontology (GO) terms using the following databases: Functional Categories (UP_KEYWORDS); gene ontology Biological Process (GOTERM_BP_FAT), Cellular Component (GOTERM_CC_FAT); Molecular Function (GOTERM_MF_FAT); General Annotations (SP_COMMENT); Pathways (BIOCARTA; KEGG PATHWAY); Protein Domains (INTERPRO); and Protein Interactions (UCSC_TFBS). The results generated by DAVID were summarized in a tabular format.

To compare transcriptome changes induced by PGE2 and E2 treatment with these associated with the pregnancy the expression signals of DEGs identified in endometrial samples collected from gilts treated with E2 and PGE2 were matched with the expression signals of DEGs identified in endometrial samples collected from gilts on day 12 of pregnancy and the estrous cycle [13]. Likewise, the expression signals of DEGs identified in endometrial samples collected from gilts on day 12 of pregnancy and the estrous cycle [13] were matched with expression signals of genes in dataset generated for endometrial samples collected from gilts treated with PGE2 and E2. Generated matrix of matched expression signals was then analyzed by hierarchical clustering using Pearson correlation. Common and group-specific DEGs were identified using jVenn plug in (jvenn.toulouse.inra.fr; [32]) and summarized in a Venn diagram and tabular format.

Topp Cluster software [33] was used to identify gene ontologies (GOs) enriched by DEGs identified in endometrial samples treated with PGE2 and E2 (compared to placebo) and

compared to GOs identified in porcine endometria treated with E2 alone and in endometrial samples collected on day 12 of pregnancy (when compared to day 12 of the estrous cycle; [13]). Results were summarized in a tabular format and visualized as a relationship network.

Ingenuity Pathway Analysis (IPA; v. 01.12; Qiagen, Redwood City, CA, USA) software was used to compare canonical pathways, upstream regulators and bio functions associated with pregnancy-specific, E2- and PGE2+E2-affected DEGs. Terms with prediction Z-score higher than 2 or lower than −2 may be regarded as significant [34]. The results of the performed analyzes were summarized in tabular and in graphic formats.

1.4.Validation of microarray results

The results of endometrial transcriptome profiling by expression microarrays were verified by qPCR (real-time RT-PCR) for following genes: caspase 3 (*CASP3*); deleted in malignant brain tumors 1 (*DMBT1*); growth differentiation factor 15 (*GDF15*); kelch like family member 14 (*KLHL14*); lysophosphatidic acid receptor 3 (*LPAR3*); mucin 4, cell surface associated (*MUC4*); prosaposin isoform A (*PSAP*); serpin family B member 7 (*SERPINB7*); solute carrier organic anion transporter family member 2A1 (*SLCO2A1*); secreted phosphoprotein 1 *(SPP1*); transient receptor potential cation channel subfamily V member 6 (*TRPV6*) and Wnt family member 5A (*WNT5A*). Quantitative PCR reactions were performed using TaqMan assays (Thermo Fisher Scientific; Supplemental Table 1); accordingly to the manufacturer's protocol or using oligonucleotide primers and SYBR Green (Thermo Fisher Scientific; Supplemental Table 1). Briefly, reverse-transcribed cDNA (3.5 µL) was added to the reaction mixture: 12.5 µL Power SYBR Green master mix, 2.5 µL of each sense and antisense primer (1 µM; Supplemental Table 1), and 4 µL of nuclease-free water. For all analyzed genes except WNT5A, the PCR programs were performed as follows:

initial denaturation (95 °C, 10 min) followed by 36 cycles of denaturation (95 °C; 15 s), annealing and elongation (60 °C; 1 min). For WNT5A following PCR program was applied: 36 cycles of: denaturation (15 s at 95°C), annealing (30 s at 59°C) and elongation (60 s at 72°C). After PCR using SYBR Green, melting curves were acquired by gradual increases in temperature from 60 to 95 °C to ensure that a single product was amplified in the PCR reaction. All qPCR reactions were performed with Applied Biosystems 7900HT Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). Gene expression was calculated using real-time PCR Miner software [35]. Stability of the reference genes in the porcine endometrium was assessed using the statistical algorithm Normfinder 2.0 [36]. Six reference genes were analyzed: *ACTB*, *GAPDH*, *HMBS*, *HPRT1*, *PPIA*, and *RPL13A*. For normalization of the results, the geometrical mean of the most stable pair of reference genes (*RPL13A* and *GAPDH*) was used. The direct local effect of E2, PGE2 and PGE2 administered simultaneously with E2 on endometrial gene expression was studied by comparison of mRNA levels in the endometrial samples from the hormone-treated horns to its expression in the samples from the placebo-treated horns of each gilt. The local indirect effects of E2, PGE2 and PGE2 administered simultaneously with E2 on endometrial gene expression were studied by comparison of mRNA levels in endometrial samples from the experimental group to the levels in the control group (placebo infusions into both horns).

2.6. Statistical analyses

To determine statistical significance of gene expression difference in the porcine endometrium on day 12 of pregnancy compared to day 12 of the estrous cycle the T-test was applied. Two-way ANOVA followed by the Bonferroni post-test was used to analyze the effect of E2 and PGE2 acting alone or simultaneously with E2 on gene expression in endometrial samples collected from in vivo experiments. Differences were considered as

statistically significant at the 95% confidence level ($p<0.05$). All statistical analyses were conducted using GraphPad PRISM v. 6.0 software (GraphPad Software Inc., San Diego, CA, USA).

2. RESULTS

2.1.Porcine endometrial transcriptome profiling using microarrays

Global gene expression profiling in porcine endometrium towards PGE2 treatment and simultaneous treatment with PGE2 (200 µg/ infusion) and estradiol-17β (33.3µg / infusion) in vivo compared to placebo (saline) treatment was determined using the porcine Agilent microarray assay. The numbers of detected probes passing the filters were as follows: 37 408 (PGE2-treated vs. placebo-treated) and 38 267 (E2+PGE2-treated vs. placebo-treated). Pairwise distance analysis based on all detectable probes revealed that endometrial samples treated with PGE2 represented mainly random grouping (Supplemental Figure 1A) whereas endometrial samples treated with PGE2 together with E2 represented mainly treatment grouping with a slight bias observed in the heatmap (Supplemental Figure 1B).

Comparing the effect of simultaneous infusion of PGE2 and E2 using LIMMA package, 4487 probes displayed significant differences in expression signal intensities (fold change </>1.5; $p < 0.01$; false discovery rate (FDR) = 5%). Collapsing the results from the probe level to the gene level revealed 2413 differentially expressed genes (DEGs; Figure 1). No probes expressing significant differences in expression signal intensity (fold change </>1.5; $p < 0.01$; false discovery rate (FDR) = 5%) were detected for endometrial samples treated with PGE2. The results of statistical analysis of microarray data are summarized in Supplemental Table 2. The Pearson correlation performed for DEGs detected in endometrial samples treated simultaneously with E2 and PGE2 revealed treatment grouping of the analyzed samples with two main distinct clusters of genes with uniform distribution of

expression signals (Figure 1). In the endometrial samples collected from horns receiving hormonal infusion (33.3 μg of E2 + 200 μg of PGE2/infusion), the five DEGs showing the highest upregulation in the pairwise comparison "E2+PGE2-treated horns" of the experimental group vs. "placebo-treated horns" of the control group were: S100 calcium-binding protein A8 (*S100A8*; 75-fold), *SERPINB7* (47-fold), S100 calcium-binding protein A9 (*S100A9;* 44-fold), aconitate decarboxylase 1 (*ACOD1*; *IRG1*; 33-fold) and S100 calcium-binding protein (*S100A12*; 26-fold) whereas the five DEGs showing the highest downregulation were: interleukin 24 (*IL24;* -31-fold), opticin (*OPTC*; -15-fold), ATPase H+ transporting V1 subunit G3 (*ATP6V1G3*; -14-fold), C1q and TNF related 3 (*C1QTNF3*; -13-fold) and regenerating family member 3 gamma (*REG3G*; -12-fold). In Figure 2 top ten differentially regulated genes detected in porcine endometrium towards E2+PGE2 treatment were compared with top ten differentially expressed genes identified in porcine endometrium treated with E2 alone (33.3 μg/infusion) and top ten DEGs identified in endometrial samples collected from gilts on day 12 of pregnancy [13].

2.2.Functional Annotation of Microarray Data

Functional terms enriched for up-regulated and down-regulated DEGs detected in endometrial samples treated with E2 together with PGE2 were identified by using the DAVID. For up-regulated DEGs DAVID identified 144 annotation clusters (enrichment score 37.2–1.3) whereas for down-regulated genes 49 annotation clusters (enrichment score 5.6–1.3) were identified.

The most interesting functional terms of overrepresented annotation clusters enriched for up-regulated genes were related to focal adhesion, cell migration, glucose metabolic process, activation of innate immune response and reproductive system development (Table

1). For pregnancy-associated down-regulated genes, the most interesting enriched terms were related to regulation of transcription and RNA biosynthetic processes, cell morphogenesis and histone acetylation, calcium transport and BMP signaling pathway (Table 1). The complete results from the functional annotation clustering performed for pregnancy-associated DEGs are summarized in Supplemental Table 3.

2.3. *Modulatory effect of PGE2 acting simultaneously with estradiol-17β compared with the effect of estradiol-17β and day 12 of pregnancy on endometrial transcriptome in the pig*

Using jVenn tool we compared the lists of up- and down-regulated genes identified in the endometrial transcriptome profiling. Comparison analyses revealed that PGE2 administered into the uterine lumen together with E2 stimulated expression of 83 genes that were found to be up-regulated in porcine endometrium on day 12 of pregnancy whereas E2 administered alone stimulated 20 genes which were up-regulated in day 12 of pregnancy endometrial samples (Fig. 3A). PGE2 acting simultaneously with E2 lowered the expression of 40 genes that were also found to be down-regulated in endometrial samples collected on day 12 of pregnancy whereas estradiol-17β acting alone lowered the expression of 10 genes that were found to be down-regulated in endometrium on day 12 of pregnancy (Fig. 3B). Results of comparison analyses are presented in Venn diagrams in Figure 3. Complete results containing lists of DEGs are summarized in tabular format in Supplemental Table 4.

To compare the changes in endometrial transcriptome evoked by E2 and PGE2 acting simultaneously with E2 to those observed on day 12 of pregnancy we used Pearson correlation analysis. We observed that the intrauterine infusions of either E2 and PGE2 together with E2 resulted in grouping of samples close to samples collected from gilts on day 12 of pregnancy whereas placebo-treated samples were grouped together with endometrial

samples collected on day 12 of the estrous cycle. However, the scale of changes within the endometrial transcriptome was much larger in the endometrial samples treated with PGE2 and E2 (Figure 4A) than in samples treated only with E2 (Figure 4B).

2.4.Functional analysis of transcriptome changes evoked by synergistic effect of PGE2 and E2 in vivo

Using ToppCluster tool we compared gene ontology terms (Biological Process, Molecular Function, Cellular Component) and pathways enriched for DEGs identified in the endometrial samples treated in vivo with PGE2+E2, E2 alone and for DEGs identified in the endometrial samples collected on day 12 of pregnancy. Results of this analysis showed that the majority of identified enriched terms were shared by all analyzed groups (Day 12 of pregnancy, PGE2 acting simultaneously with E2 and E2 administered alone). However, a number of terms and pathways were found to be enriched only for DEGs identified in the endometrial samples on day 12 of pregnancy and in the endometrial samples treated with PGE2 together with E2. These were related to embryo implantation, fibroblast migration, water homeostasis or regulation of extent of cell growth, organ growth, serine/threonine-protein kinase PLK1 signaling, collagen biosynthesis and others. The most interesting identified terms were summarized in graphical format in Figure 5. Complete results were summarized in tabular format in Supplemental Table 5.

Using Ingenuity Pathway Analysis we performed Comparison Analyses for Canonical Pathways and Diseases and Biofunctions enriched for DEGs identified in analyzed groups (Day 12 of pregnancy, E2+PGE2 and E2 alone). We compared Z-score values of identified terms. We found that PGE2 administered into the uterine lumen together with E2 augmented a Z-score value for such pathways as: GP6 signaling pathway, integrin signaling, glycolysis,

(vascular endothelial growth factor (VEGF) signaling and VEGF family ligand-receptor interactions, prolactin signaling, eNOS signaling and many others (Fig. 6A). Comparing diseases and functions it was demonstrated that PGE2 acting simultaneously with E2 modulated a Z-score value for such functions as cell movement and migration, inflammatory response, transport of molecules, fibrogenesis, proliferation of connective tissue cells, apoptosis and many others (Fig. 6B). The most interesting terms were summarized in Figure 6A and B. Complete results of comparison analyses performed in IPA software are summarized in Supplemental Tables 6 and 7.

Comparing results of Functional Annotation Clustering performed in DAVID for DEGs identified in endometrial samples treated either with PGE2 together with E2 and treated only with E2 we selected group-specific terms enriched for DEGs in analyzed gene sets (Fig. 6C). The most interesting terms enriched for genes up-regulated due to modulatory effect of synergistic action of PGE2 and E2 were related to tissue morphogenesis, response to insulin, metallopeptidase activity, vasodilation, cell adhesion mediated by integrins and others whereas terms enriched for down-regulated genes were related to circadian entrainment, regulation of histone methylation or regulation of RNA biosynthetic process.

2.5.Validation of microarray results

The expression of the following DEGs identified in analyzed groups (Day 12 of the estrous cycle/ pregnancy, PGE2, E2 or PGE2 administered together with E2 was validated using quantitative PCR (real-time RT-PCR; qPCR): *CASP3*, *DMBT1*, *GDF15*, *KLHL14*, *LPAR3*, *MUC4*, *PSAP*, *SERPINB7*, *SLCO2A1*, *SPP1*, *TRPV6* and *WNT5A*. Overall, qPCR analysis confirmed the pattern of gene expression detected using expression microarrays in analyzed groups (Supplemental Table 8). The expression of *GDF15* and *WNT5A* was found to

be lowered either by E2 administered alone and acting simultaneously with PGE2 ($p<0.05$; Fig. 7C and L). Intrauterine infusions of E2 and E2 administered together with PGE2 resulted in elevated expression of *DMBT1, TRPV6*, and *SERPINB7* genes ($p<0.05$; Fig. 7B, K and H). Administration of PGE2 together with E2 modulated down-regulation of *KLHL14* and up-regulation of *SPP1* genes ($p<0.05$; Fig. 7 D and J). Expression of *LPAR3*, *MUC4, PSAP*, and *SLCO2A1* was found to be stimulated only by synergistic action of PGE2 with E2 ($p<0.05$; Fig. 7 E, F, G and I). Interestingly, expression of *CASP3* was found to be regulated only by PGE2 administered alone but not in other treatments ($p<0.05$; Fig. 7A). Expression patterns of genes analyzed in endometrial samples collected on day 12 of the estrous cycle and pregnancy was convergent to those observed in endometrial samples collected from in vivo experiment.

3. DISUCSSION

During pregnancy establishment, the endometrium undergoes rapid morphological and functional transitions to develop receptivity for embryo implantation. These are preceded by changes in the global gene expression profile. Previous reports on endometrial transcriptome profiling in the pig revealed a large number of differentially expressed genes involved in multiple processes vital for pregnancy establishment and development [8–11, 37]. This is the first report studying the synergistic effect of E2 and PGE2 — the hormones which have been recently suggested as two maternal recognition signals [1, 12, 20, 21]. The present study involves a novel approach of studying the effect of hormones administered in vivo directly into the uterine lumen, which imitates the secretion of E2 and PGE2 by conceptuses during the time corresponding to the maternal recognition of pregnancy. Recently, we reported that estradiol-17β, which is the main embryonic signal in the pig, is responsible for major changes occurring in the endometrial transcriptome during the maternal recognition of pregnancy [13].

We also indicated that prostaglandin E2 is a key factor involved in controlling reproductive function at the embryo–maternal interface (reviewed in [1]). In the present study, we evidenced for the first time that PGE2 acting synergistically with E2 in the porcine endometrium in vivo, induces substantial changes in global gene expression profile. Utilizing an in vivo model of hormonal infusions directly into the uterine lumen, we demonstrated that PGE2 administered together with E2 altered the expression of 2413 genes, whereas administration of E2 alone altered the expression of 1957 genes [13]. In further analyses we applied Venn diagrams to compare DEGs common to in vivo groups (E2, and E2+PGE2), and day 12 of pregnancy served as a referential group. These analyses revealed that administration of PGE2 together with E2 resulted in a higher number of DEGs that were also identified in endometrial samples collected from gilts on day 12 of pregnancy compared with DEGs altered by E2 acting alone. However, administration of only PGE2 had no effect on the global gene expression profile in the porcine endometrium. These results clearly indicate the modulatory effect of PGE2 augmenting the changes induced by E2. Our data are consistent with conclusions based on a recent study indicating that both PGE2 and E2 act as maternal recognition signals [12, 21]. However, conceptus production of estrogen is essential for successful maintenance of pregnancy beyond day 30 because prostaglandins of conceptus and endometrial origin are not successful in compensating for ablated estrogen production at this time [21]. However, porcine conceptuses are not the only source of estrogens in the uterine environment. Active estrogen synthesis and secretion by the myometrium and endometrium have been indicated as alternative sources for this signal for recognition of pregnancy in the pig [38]. Similarly, our results confirmed that PGE2 is also synthesized and secreted in the porcine endometrium and its endometrial synthesis and secretion is stimulated by estradiol-17β and PGE2 of conceptus origin [14, 16]. Hence, based on the aforementioned studies, it can be speculated that impaired production of E2 and/or PGE2 by porcine conceptuses is

compensated by endometrial E2 and/or PGE2 synthesis and secretion, which in the absence of conceptus production of either E2 or PGE2, is sufficient to support the establishment of pregnancy and conceptus maintenance until days 30-35 of pregnancy.

4.1. Functional analysis of synergistic effect of E2 and PGE2 on global gene expression profile

Functional annotation analyses of DEGs affected by the synergistic effect of PGE2 and E2 identified the processes which have been described to be enriched either for pregnancy-specific or E2-affected DEGs: extracellular secretion, vasculature development, cell adhesion, migration and proliferation, regulation of immune response, regulation of transcription, and many others [8–11, 13]. Interestingly, analyses performed by Ingenuity Pathway Analysis software allow for comparison of activation/inhibition prediction factor (Z-score) calculated for identified terms (canonical pathways, diseases, and bio-functions). Our results revealed that simultaneous administration of PGE2 and E2 modified (enhanced or reduced) the value of the Z-score calculated for several terms. Comparing the canonical pathways related with DEGs we found that the addition of PGE2 increased the Z-score calculated for pathways related with processes involving pregnancy establishment on day 12 such as angiogenesis (VEGF signaling, VEGF ligand receptors family, eNOS signaling), hormone action (prolactin signaling), tissue remodeling (actin cytoskeleton signaling), and energetic processes (glycolysis, gluconeogenesis, and insulin receptor signaling). Similarly, simultaneous administration of PGE2 with E2 modulated the Z-score value calculated for bio-functions involved in the embryo–maternal dialogue, as well as those vital for preparation of the endometrium for embryo attachment, such as cell movement/migration, inflammatory

response, vasculogenesis, transport of molecules, cell proliferation, secretion of molecules, necrosis, and apoptosis.

Comparison analyses of identified gene ontology terms, in addition to comparison of activation/inhibition prediction factors, allowed the processes related to the synergistic action of PGE2 with E2 to be extracted. Using ToppCluster we identified PGE2+E2-specific GO terms related to biological processes (i.e., organ growth, embryo implantation, water homeostasis), cellular components (chromosome centromeric regions, cyclin-dependent protein kinase holoenzyme complex, filopodium membrane), molecular functions (fibrinogen binding, G-protein coupled glutamate receptor binding or PDGF binding). Our findings also suggest that synergistic action of PGE2 together with E2 affects the pathways involved in tissue remodeling (collagen biosynthesis and modifying enzymes) and cell division (PLK1 signaling events, aurora B signaling). Polo-like kinase (PLK) is a highly conserved serine/threonine protein kinase with a highly homologous serine/threonine kinase domain at its N-terminus, which regulates PLK activity and subcellular dynamics at the C-terminus and targeted polo-box domain (PBD). PLK proteins have been described to regulate many key steps in the cell cycle, including the formation of bipolar spindles, chromosome segregation, late regulation of complexes, and cytokinesis [39]. Aurora B kinase is a protein that functions in the attachment of the mitotic spindle to the centromere and has been reported as a necessary factor in cytokinesis [40].

In the present study we also compared the results from functional annotation analysis performed in DAVID separately for E2– and E2+PGE2-affected DEGs. Removing the processes identified for both analyzed groups allowed the processes characteristic only for simultaneous action of PGE2 and E2 to be extracted. Terms identified for up-regulated genes were related, among others, to tissue morphogenesis, cell adhesions, development of connective tissue, insulin resistance, regulation of endothelial cell proliferations, and cytokine

binding, whereas terms enriched for down-regulated genes identified for the synergistic effect of E2 and PGE2 were related to DNA binding, regulation of RNA biosynthesis, or circadian entrainment. In summary, using various bioinformatics tools we obtained consistent results evidencing that PGE2 acting simultaneously with E2 strengthened the effect of E2 on changes in the global gene expression profile in the porcine endometrium, affecting the processes related to changes in tissue rearrangements, immune response, cell cycle, and energy balance, which are critical for successful pregnancy establishment. Moreover, changes in the endometrial transcriptome induced by the synergistic action of PGE2 with E2 better resembled the changes identified in the porcine endometrium during early pregnancy compared to the E2 action alone.

4.2. The local direct and indirect effects of E2 and E2 acting together with PGE2 on the endometrial expression of genes involved in pregnancy establishment and development

Validation of microarray results using qPCR revealed that, in contrast to the effect of E2, only the synergistic action of PGE2 and E2 on the endometrial gene expression of *LPAR3*, *MUC4*, *PSAP*, and *SLCO2A1* reflected the effect of pregnancy on day 12 on the expression of these genes. PGE2 augmented the stimulating effect of E2 on *SPP1* expression in the endometrium corresponding to expression on day 12 of pregnancy. PGE2 acting together with E2 altered the expression of *DMBT1, GDF15, KLHL14, SERPINB7, TRPV6*, and *WNT5A* genes to the similar extent as E2 administered alone and the pregnancy effect.

The most frequent processes enriched for pregnancy-specific, E2–, and E2+PGE2–affected DEGs were found to be related to ion and molecule transport, immune response, tissue remodeling, cell adhesion, proliferation, and differentiation, which are crucial for pregnancy establishment in pigs. Comparison of functional annotation analyses revealed that PGE2 accompanied the E2 in regulation of processes related to the transport of molecules.

These processes support a proper supply of endometrial tissue with nutrients and factors regulating its function. In the present study, we demonstrated that the synergistic action of PGE2 with E2 in vivo increased the gene expression of *SLCO2A1*, which serves as a prostaglandin transporter. Our recent reports indicated a vital role for prostaglandins (PGE2 and PGF2α) in the establishment and development of pregnancy [1, 22, 41–44]. The stimulating effect of E2 acting simultaneously with PGE2 on the expression of *SLCO2A1* is the more interesting result, especially when we take into consideration our description of the role of E2 and PGE2 in the mechanism of the PGE2-positive feedback loop in the porcine endometrium [16].

The molecules acting at the embryo–maternal interface to exert their actions require mechanisms transducing their signaling. A number of intracellular processes enriched for identified DEGs were related to signaling involving calcium ions. During the peri-implantation period in pigs the elevated concentration of calcium ions in the uterine lumen has been observed [45]. Recently, we reported the stimulating effect of E2 on the *TRPV6* gene [13], which has been implicated in mediating calcium ion uptake [46]. Moreover, greater expression of *TPRV6* in the porcine endometrium during early pregnancy has been described [47] and indicated as the marker of uterine receptivity. Interestingly, in the present study, we evidenced that administration of PGE2 together with E2 amplified the stimulating effect of E2 on *TRPV6* expression.

Inflammatory mediators are the key players ensuring a proper immune response of the maternal organism to developing embryos. Indeed, the processes related to immune response have been enriched for pregnancy-related endometrial DEGs [8–11, 13]. Hormones (i.e., E2), cytokines (interleukins, interferons), and prostaglandins are involved in the establishment of this proinflammatory environment (reviewed in [1] and [48]). Proteins belonging to the SERPIN family have been widely described to participate in the immunomodulation in

reproductive tissues in cattle [49]. Interestingly, the *SERPINB7* gene has been described to be up-regulated in the endometrium in reports on porcine endometrial transcriptome profiling [8, 9]. Using an in vivo model of hormonal infusions directly into the uterine lumen, we demonstrated that PGE2 administered together with E2 had a direct stimulating local effect on *SERPINB7* gene expression, which is also consistent with its expression profile in the porcine endometrium on day 12 of pregnancy [13]. Prosaposin (PSAP) is a multifunctional protein whose role has been mainly described for the nervous system [50]. However, gene ontology analyses linked *PSAP* with processes related to innate immunity. Synergistic action of E2 and PGE2 modulated the stimulating effect of E2 on *PSAP* gene expression, which was also consistent with the expression profile of these genes in day 12 of pregnancy endometrial samples. Kelch Like Family Member 14 (KLHL14) has been described to be involved in a variety of cellular mechanisms such as control of cytoskeletal organization [51], ion channel [52], transcription suppression [53], and lymphocytes B-1a development in mice [54]. Previously we reported a diminishing effect of E2 on the expression of the *KLHL14* gene in vivo [13]. In the present study, we found that PGE2 acting together with E2 also led to decreased levels of *KLHL14* mRNA content, however, this effect was not as strong as the effect of E2 administered alone. These results suggest that PGE2, by acting in addition with E2, may be involved in the modulation of the proinflammatory environment in the porcine endometrium during early pregnancy.

Results of studies of endometrial transcriptome profiling revealed a number of processes related to tissue remodeling. Indeed, during early pregnancy the endometrial tissue undergoes rapid structural and morphological changes to enable the attachment of conceptuses to its surface. These changes are orchestrated by a variety of factors [1, 48]. Mucin 4 (MUC4), a high-molecular weight glycoprotein, has been indicated to be responsible for protecting the surface of most epithelia [55]. In porcine surface and glandular epithelia its

expression is increased during the luteal phase and reduced during early proestrus [56]. Interestingly, its elevated expression has been found on days 12 and 14 of pregnancy compared to days 12 and 14 of the estrous cycle [8, 10]. Considering these findings, authors hypothesized that MUC4 may be involved in protecting the epithelial layer of the endometrium against invasive trophoblast. Recently, we reported no effect of E2 (33.3 µg/infusion) on *MUC4* expression in vivo [13]. However, in the present study, we found that simultaneous administration of PGE2 and E2 into the uterine lumen significantly increased the endometrial expression of the *MUC4* gene, which was consistent with its elevated expression on day 12 of pregnancy [13]. Another DEG that has been linked with GO related to tissue remodeling is growth differentiation factor 15 (GDF15), which is a member of the transforming growth factor beta (TGFB) superfamily [57]. It has been described to be abundantly expressed in the human placenta [58, 59]. Several reports indicate that GDF15 may be involved in actin cytoskeleton reorganization and remodeling [60]. It has been also suggested to be a necessary molecule for the maintenance of pregnancy in humans [61] and rats [62]. In our previous report we documented that E2 lowered the expression of *GDF15* in the porcine endometrium in vivo [13]. Herein, we evidenced that synergistic action of E2 together with PGE2 decreased the levels of *GDF15* mRNA in a direct local manner. GDF15 participates in placenta development via promoting trophoblast cell invasion [63]; therefore, we assume the hypothesis that the down-regulation of *GDF15* in the porcine endometrium in response to E2 and E2 acting together with PGE2 may be involved in the mechanisms protecting the porcine endometrium from invasion of trophoblast into the uterine wall.

Cell proliferation, adhesion, migration, and differentiation are the processes accompanying tissue remodeling. In our previous report, using porcine and human in vitro models we evidenced that PGE2 affecting integrin receptors, expression of focal adhesion kinase 1, and intercellular adhesion molecule proteins supported trophoblast cell adhesion to

extracellular matrix proteins [20]. Functional annotation analyses performed in the present study for transcriptome profiling results revealed that cell adhesion was strongly enriched for DEG identified in the endometrial samples treated simultaneously with E2 and PGE2. Secreted phosphoprotein 1 (SPP1) has been suggested to be the main contributor to stable adhesion during the peri-implantation period [64]. Binding of SPP1 to these various receptors is involved in diverse effects, including cell-to-cell and cell-to-extracellular matrix adhesion, endothelial cell chemotaxis, and endothelial and epithelial cell survival [65]. In the present study, we evidenced that E2 administered alone stimulated expression of the *SPP1* gene in an indirect local manner – both in placebo and E2-treated horns compared to its expression in endometrial samples collected from animals of the control group (placebo infusions into both uterine horns). It is consistent with findings that systemic administration of estrogens induces endometrial expression of SPP1 [66]. Interestingly, presented results indicate that PGE2 enhanced the stimulating effect of E2 on *SPP1* expression. These results strongly support our hypothesis that PGE2 is a molecule involved in cell adhesion [20]. DMBT1 has been described to be involved in cell proliferation and differentiation. Results of studies on primate and rodent uteri revealed that DMBT1 is a protein induced by E2 and was localized to the epithelial layer of the endometrium. It has been also suggested to be involved in endometrial growth and/or differentiation [67]. Recently, we demonstrated that expression of *DMBT1* is up-regulated in the porcine endometrium on day 12 of pregnancy and that this up-regulation was induced by E2. In the present study, we found that simultaneous administration of E2 together with PGE2 led to up-regulation of *DMBT1* gene to the same extent as E2 administered alone. Lysophosphatic acid (LPA) is an important factor influencing several physiological processes involved in the reproductive function of the female (reviewed in [68]). Its receptor (LPAR3) has been proposed to be a uterine receptivity marker critical for embryo migration and spacing in mice [69]. LPA promoted proliferation, migration, and

differentiation of porcine trophoblast cells by activating the ERK1/2-P90RSK-RPS6 and P38 pathways. Due to this fact it has been suggested that LPA-LPAR3 signaling is involved in the development of the trophoblast during early pregnancy in pigs [70]. Recently, we reported the stimulating effect of E2 on endometrial *LPAR3* expression in the pig in vivo [13]. In the present study, however, we found that simultaneous administration of E2 together with PGE2 into the uterine lumen increased *LPAR3* expression to a higher extent than E2 administered alone. These results are also consistent with the elevated expression of *LPAR3* detected in the porcine endometrium on day 12 of pregnancy [13].

Apoptosis is a process closely related to tissue remodeling. The morphological changes observed in cells undergoing apoptosis are the final result of the activation of specific enzymes known as "caspases". Caspases-2, -3, and -10 were reported to be exclusively associated with apoptosis. Activation of caspase-3 has been linked with the phenomenon of irreversible cell death [71]. In the present study, PGE2 administered alone increased the *CASP3* mRNA content, which was also consistent with the elevated expression of *CASP3* in the endometrial samples collected from gilts on day 12 of pregnancy [13].

Developmental changes of the porcine endometrium are also associated with the expression of *WNT5A* gene as described earlier [72, 73]. Studies performed in the human endometrium indicate that WNT signaling may support the glandular function and stromal transformation [74]). Our recent results revealed that intrauterine infusions of E2 resulted in down-regulation of the *WNT5A* gene in the porcine endometrium [13]. Administration of E2 together with PGE2 also resulted in lowered content of *WNT5A* mRNA, which is consistent with the lower expression of the *WNT5A* gene detected in the porcine endometrium on day 12 of pregnancy (compared to day 12 of the estrous cycle). However, to determine the biological relevance of *WNT5A* down-regulation in the porcine endometrium further studies are required.

4.3. Conclusions

Summarizing, the results of transcriptome profiling and gene expression analyses fully evidenced that PGE2 augments the effect of E2 on changes in the endometrial transcriptome during early pregnancy in the pig. Our present findings suggest that the synergistic action of estradiol-17β and prostaglandin E2 resembles the pregnancy effects on the endometrial transcriptome better than E2 administered alone. The results presented in this study strongly support our hypothesis that both E2 and PGE2 act as mediators of conceptus signaling involved in processes important for pregnancy establishment and development in the pig.

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FIGURES LEGENDS

Figure 1. Changes in global gene expression profile detected in the endometrial samples treated simultaneously with E2 (33.3 µg/ infusion) and PGE2 (200 µg/ infusion). Data are shown as a heatmap of hierarchical clustering (Pearson correlation) of the normalized values of the intensity of the expression signals of probes detected as significantly changed (log fold change >0.585; p nominal <0.01; false discovery rate (FDR) = 5%) in the analyzed groups. CTRL-endometrial samples collected from gilts assigned to the control groups (infusions of placebo to both uterine horns), EX_C-endometrial samples collected from gilts assigned to the experimental group (uterine horns receiving placebo infusions), EX_H-endometrial samples collected from gilts assigned to the experimental group (uterine horns receiving infusions of PGE2 with E2). In the control group, each horn received intrauterine placebo infusions. In experimental group, randomly selected horns within each gilt received hormonal infusions of PGE2 (200 µg/infusion) simultaneously with E2 (33.3 µg/infusion) whereas the contralateral horn received placebo infusions.

FIGURE 1

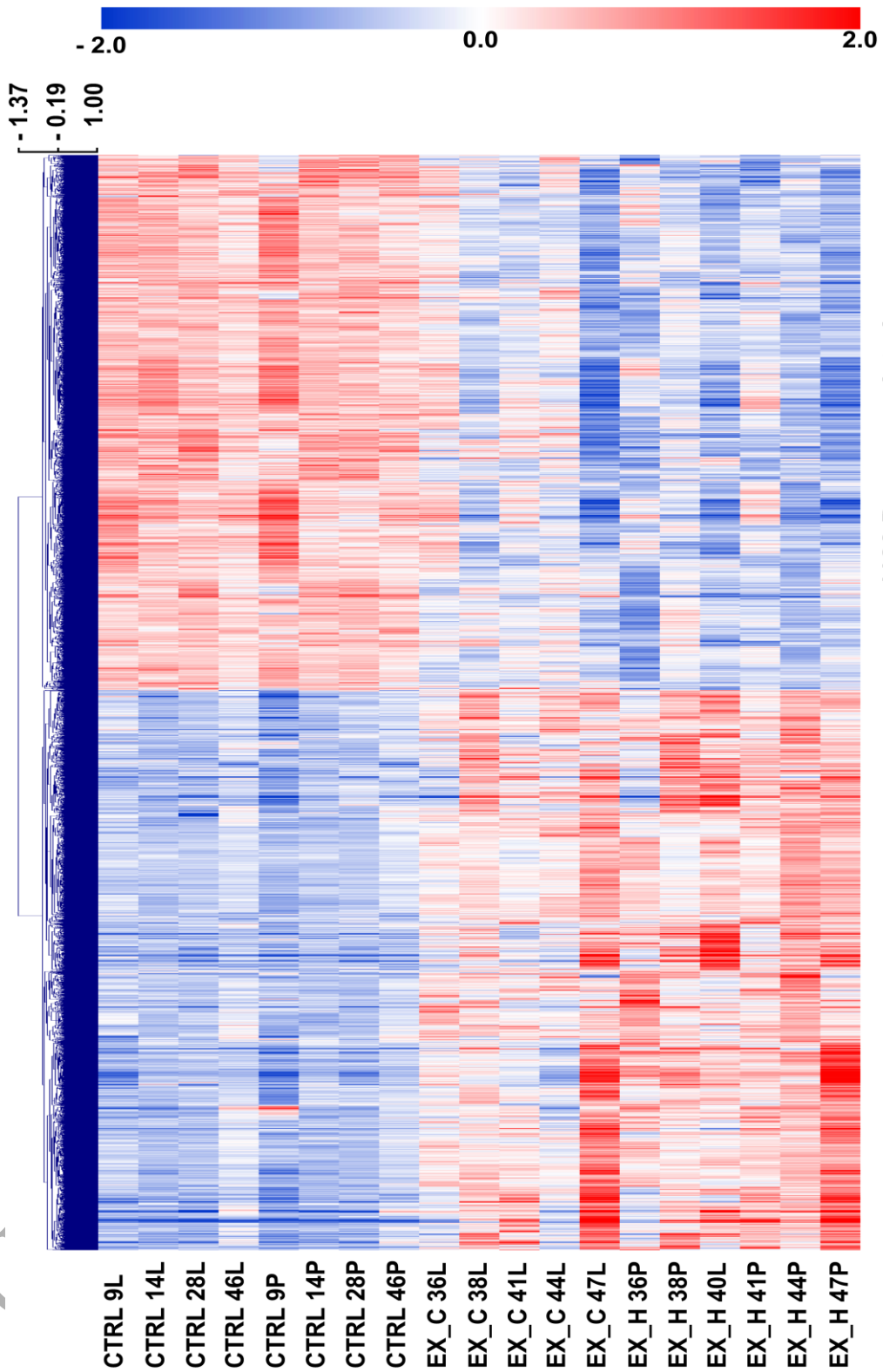


Figure 2. Heatmap of the log2 fold changes of the top 10 differentially expressed genes detected in the endometrial samples collected from gilts treated with estradiol-17β (33.3 μg/ infusion; Kaczynski et al., 2020), estradiol-17β (33.3 μg / infusion) together with prostaglandin E2 (PGE2; 200 μg/ infusion) and in the endometrial samples collected from gilts on day 12 of pregnancy [13]. The scale is from log2 fold change -5 (blue, down-regulated) to 9 (red, up-regulated). Each row represents one gene, and each column represents a comparison (E2- or E2+PGE2-treated or pregnant).

FIGURE 2

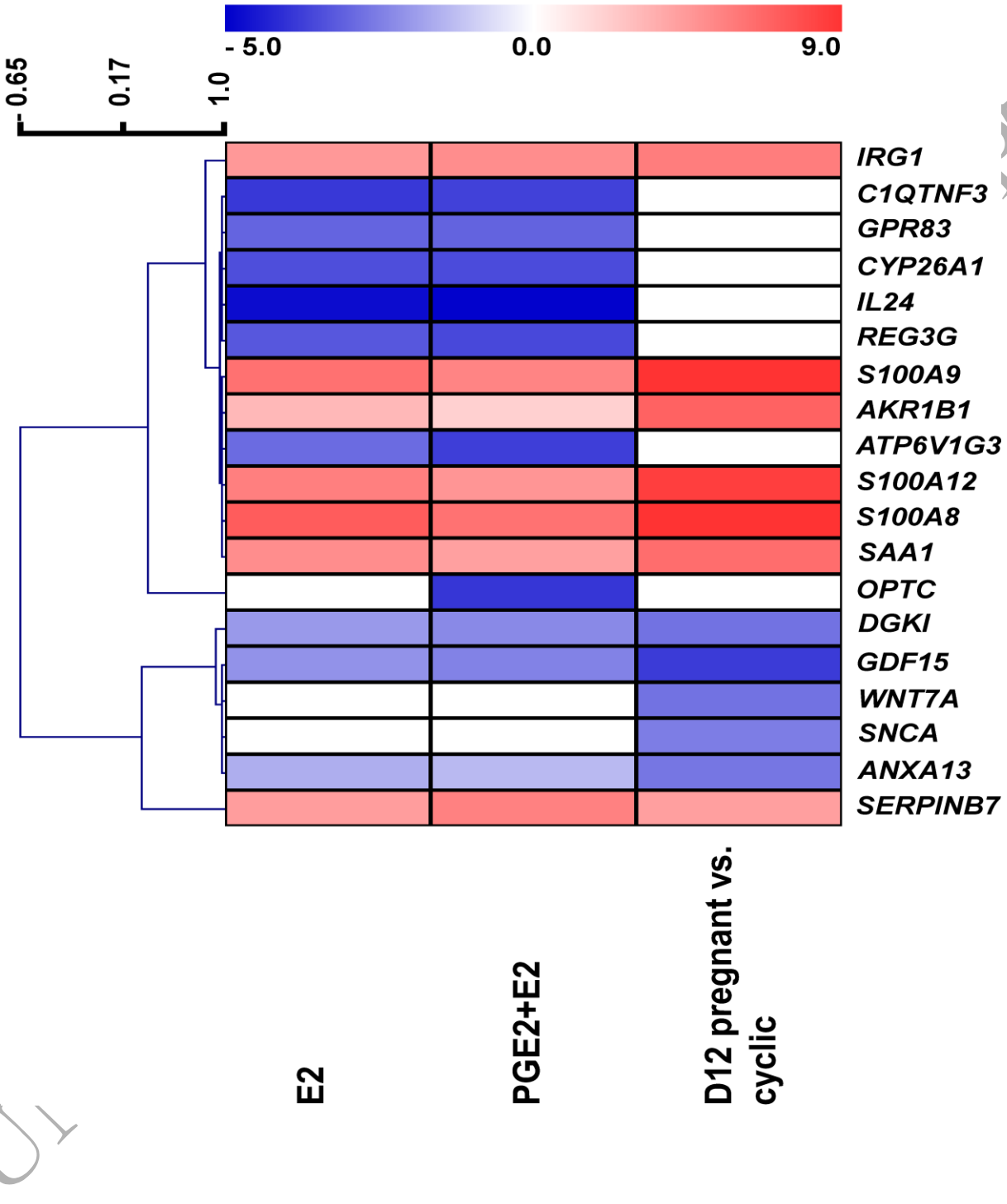


Figure 3. The results of comparison analyses of DEGs identified in endometrial samples treated in vivo with E2 and E2+PGE2 and in endometrial samples collected from gilts on day 12 of pregnancy. Comparisons were performed separately for up - (A) and down-regulated (B) genes. Tabular results containing the lists of DEGs are summarized in Supplemental Table 4.

FIGURE 3

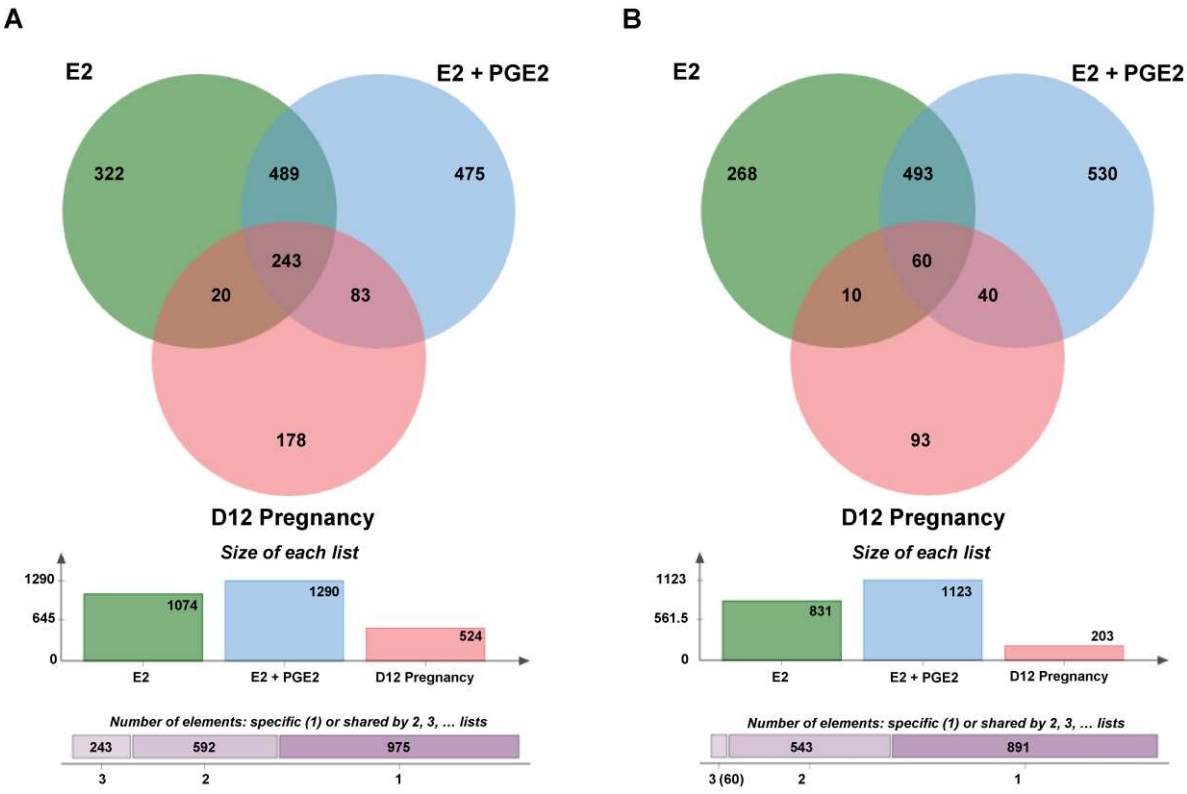


Figure 4. (A) Comparison of transcriptome changes induced by the effect of intrauterine administration of PGE2 (200 μg/infusion) together with estradiol-17β (33.3 μg of E2/infusion) with those detected in the endometrial samples collected from gilts on day 12 of the estrous cycle and pregnancy. (B) Comparison of transcriptome changes induced by the effect of intrauterine administration of estradiol-17β administered alone (33.3 μg of E2/infusion)) with pregnancy-effect on day 12 (modified from [13]). Data are shown as a heatmap of hierarchical clustering (Pearson correlation) of the normalized values of the intensity of the expression signals of probes detected as significantly changed (log fold change > 0.585; p nominal < 0.05 false discovery rate (FDR) = 5%) in analyzed groups. Cyclic-endometrial samples collected on day 12 of the estrous cycle, pregnant-endometrial samples collected on day 12 of pregnancy, CTRL-endometrial samples collected from gilts assigned to the control groups (infusions of placebo into both uterine horns), EX_H-endometrial samples collected from gilts assigned to the experimental group (uterine horns receiving PGE2+E2 or E2 infusions), EX_C-endometrial samples collected from gilts assigned to experimental group (uterine horns receiving placebo infusion).

FIGURE 4

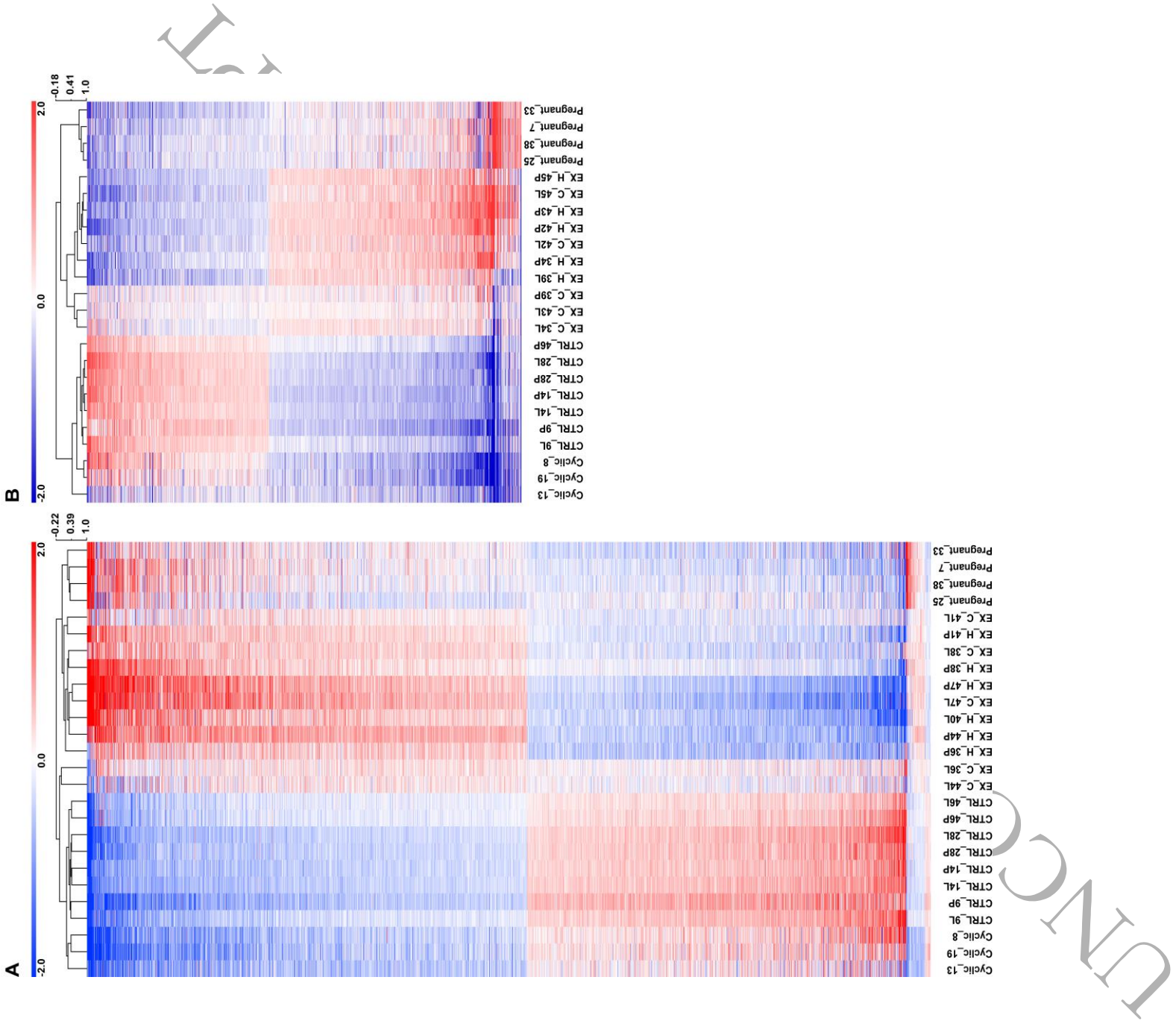


Figure 5. A functional map showing shared and group-specific functional annotation terms generated in multi-cluster gene functional enrichment analysis for differentially expressed genes (DEGs) identified in porcine endometrial samples collected on day 12 of pregnancy and treated with PGE2 (200 µg/infusion) together with E2 (33.3 µg/infusion) or with E2 alone (33.3 µg/infusion). Analysis was performed in ToppCluster software, the functional map was edited and adjusted using Cytoscape. The redundant and noninformative terms were manually removed. The complete results are presented in a tabular format in Supplemental Table 5.

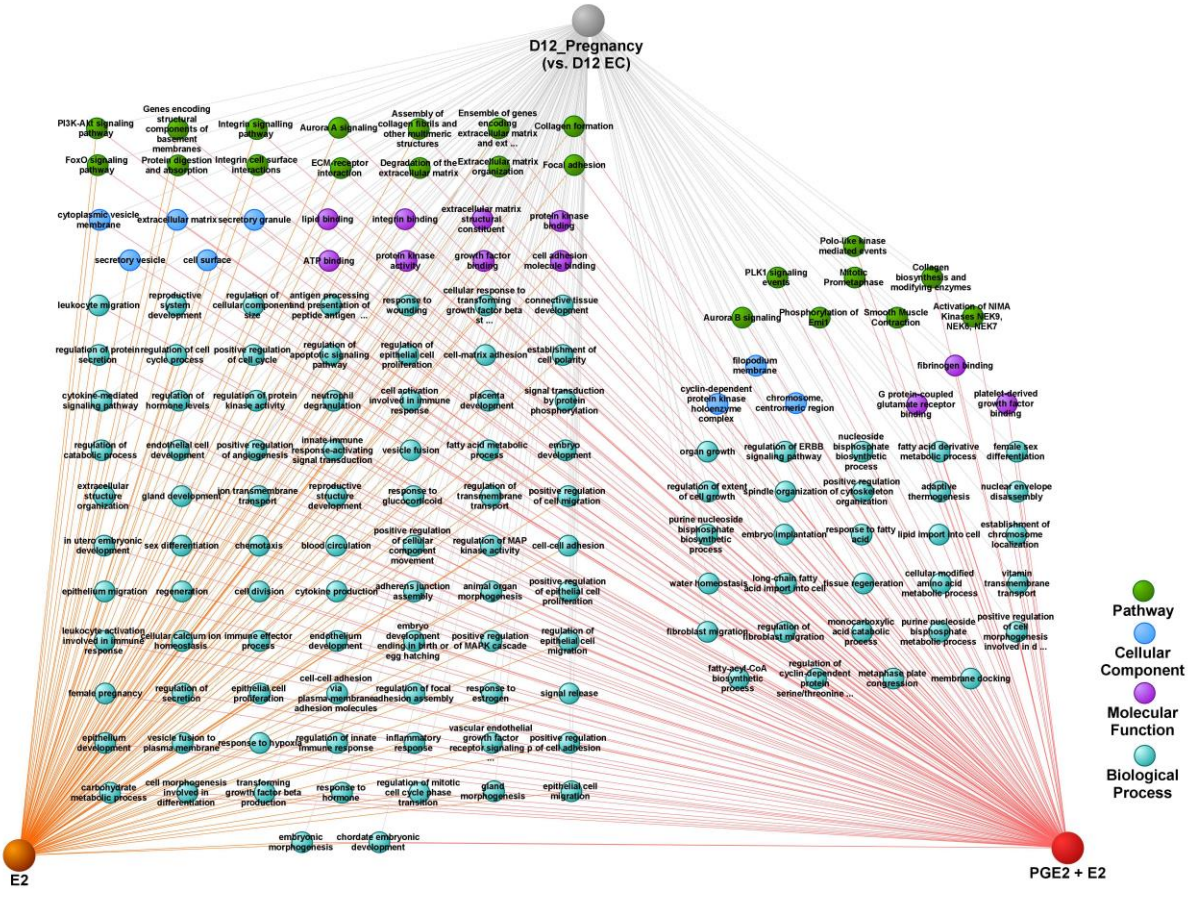


Figure 6. Results of comparison analyses performed for results generated by the Ingenuity Pathway Analysis (A) and DAVID Functional Annotation Clustering (B). Selected terms of Canonical Pathways and Diseases and Biofunctions sections (IPA) are presented together with Z-score values. A Z-score value higher than 2 means that the enriched pathway or enriched function is significantly activated or stimulated. A Z-score value lower than 2 means that the enriched pathway (A) or enriched function (B) is significantly inhibited or suppressed. Grey color indicates there is no data allowing for activation/suppression or stimulation/inhibition prediction of the enriched term. Functional annotation terms specific for modulatory effect of PGE2 acting together with E2 on global gene expression profile in the porcine endometrium are presented as bar charts generated separately for terms enriched for up- and down-regulated genes (C). Fold enrichment and -Log of p-value calculated for each term are represented by blue and orange bars, respectively.

FIGURE 6

A

Canonical Pathway	E2	E2 + PGE2	D12 Pregnant-Cyclic
GP6 Signaling Pathway	2.65	4.46	3.64
Actin Cytoskeleton Signaling	2.26	2.9	2.67
Integrin Signaling	2.53	2.77	2.84
IL-7 Signaling Pathway	0.78	2.83	2.24
Glycolysis I	2.12	2.83	N/A
IL-6 Signaling	2.65	2.79	1.67
Paxillin Signaling	1.89	2.71	2.45
ILK Signaling	2.08	2.65	2.67
VEGF Signaling	1.61	2.6	1.89
Prolactin Signaling	0.78	2.52	N/A
Cyclins and Cell Cycle Regulation	0.58	2.5	2.45
Gluconeogenesis I	1.63	2.45	N/D
Intrinsic Prothrombin Activation Pathway	N/A	2.45	2
VEGF Family Ligand-Receptor Interactions	0.73	2.4	N/A
cAMP-mediated signaling	1.79	2.34	1.89
PDGF Signaling	0.28	2.32	N/A
eNOS Signaling	0.85	2.19	2.12
Insulin Receptor Signaling	1.21	2.13	1.13
Cell Cycle Control of Chromosomal Replication	1.51	2.11	2.24
PAK Signaling	0.28	2	1.13
NF-κB Signaling	2.4	1.81	1.9
Inhibition of Matrix Metalloproteases	-0.4	-1.3	-1.3
Role of CHK Proteins in Cell Cycle Checkpoint Control	-1.1	-2.1	-2
Glutathione Biosynthesis	N/D	N/A	N/D
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	N/D	N/A	N/D
Pentose Phosphate Pathway (Oxidative Branch)	N/D	N/A	N/D
Catecholamine Biosynthesis	N/D	N/A	N/D
Serotonin and Melatonin Biosynthesis	N/D	N/A	N/D

Z-score value: -3 to 5

N/D **no canonical pathway / function detected**

N/A **no activation / inhibition prediction available**

B

Diseases and biofunction	E2	E2 + PGE2	D12 Pregnant-Cyclic
Cell movement	7.2	8.2	6.6
Migration of cells	6.4	7.5	6.1
Cell movement of blood cells	5.7	6.6	4.8
Leukocyte migration	5.6	6.3	4.6
Cell movement of leukocytes	4.5	5.5	4.1
Cell movement of neutrophils	3.3	3.9	2.4
Inflammatory response	2.9	3.7	1.3
Migration of phagocytes	N/D	3.5	4.5
Vasculogenesis	3.0	3.2	3.6
Transport of molecule	2.4	3.1	2.7
Quantity of blood cells	2.5	2.8	1.6
Neoplasia of cells	2.1	2.8	2.7
Metabolism of nucleic acid component or derivative	N/D	2.8	N/D
Formation of cytoskeleton	2.2	2.6	2.1
Cellular homeostasis	2.5	2.6	2.9
Growth of epithelial tissue	2.2	2.5	1.5
Synthesis of carbohydrate	N/D	2.5	N/D
Fibrogenesis	1.6	2.5	2.3
Aggregation of blood cells	N/D	2.4	N/D
Proliferation of cells	N/D	2.4	2.2
Growth of connective tissue	2.0	2.4	1.3
Metabolism of nucleotide	N/D	2.3	N/D
Secretion of molecule	1.9	2.3	2.2
Proliferation of connective tissue cells	1.7	2.2	1.3
Cellular infiltration	1.9	2.1	1.6
Metabolism of membrane lipid derivative	N/D	1.7	N/D
Endothelial cell development	2.1	1.7	1.8
Differentiation of connective tissue cells	3.4	1.7	0.7
Metabolism of polysaccharide	N/D	1.2	N/D
Migration of vascular endothelial cells	N/D	0.1	N/D
Cell death of connective tissue cells	0.4	-1.4	0.1
Disorder of pregnancy	N/D	-1.8	N/D
Cell death of epithelial cells	-0.2	-1.8	N/D
Necrosis	-1.8	-3.5	-3.0
Growth failure or short stature	-3.2	-3.8	N/D
Apoptosis	-2.9	-3.8	-2.3
Healing of wound	N/D	N/A	N/D
Morphology of reproductive system	N/D	N/A	N/D

Z-score value: -4 to 8

C

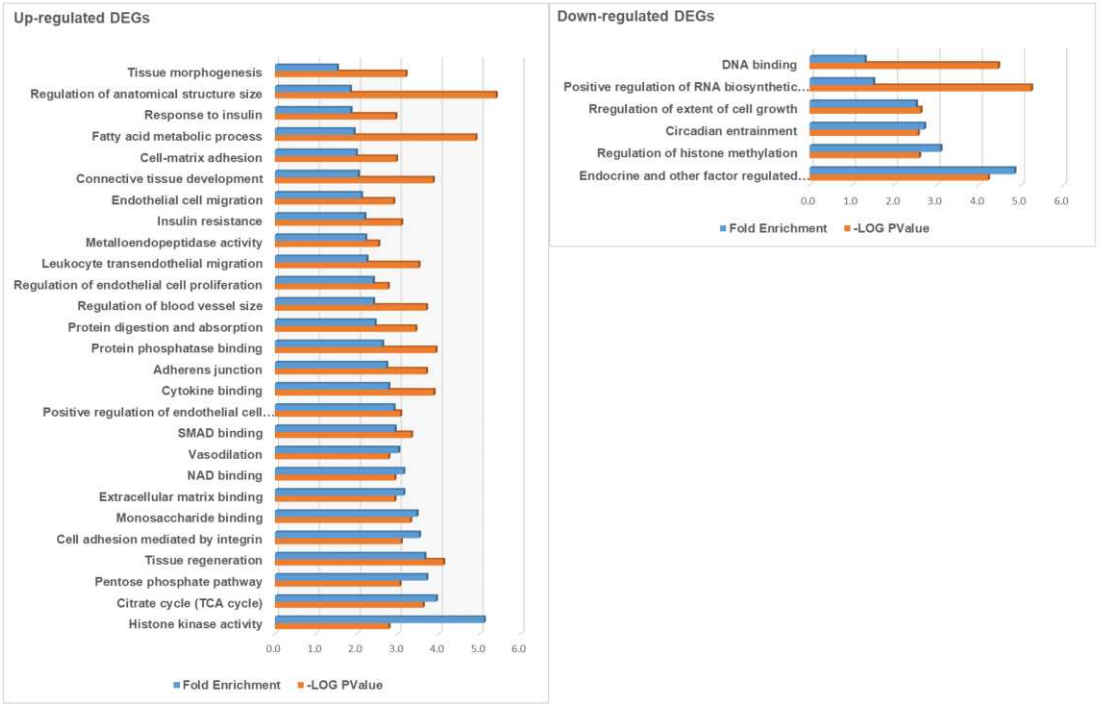
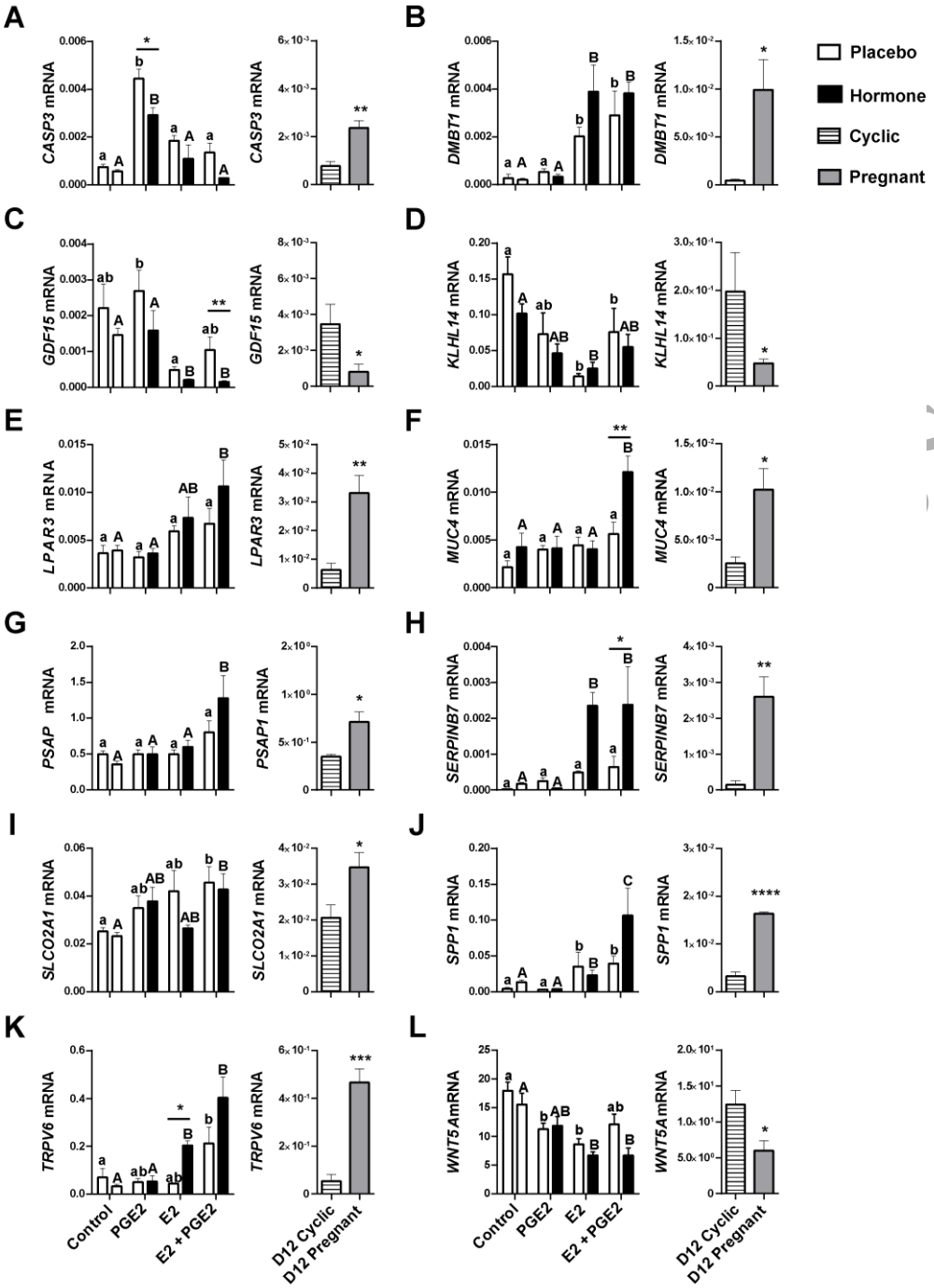
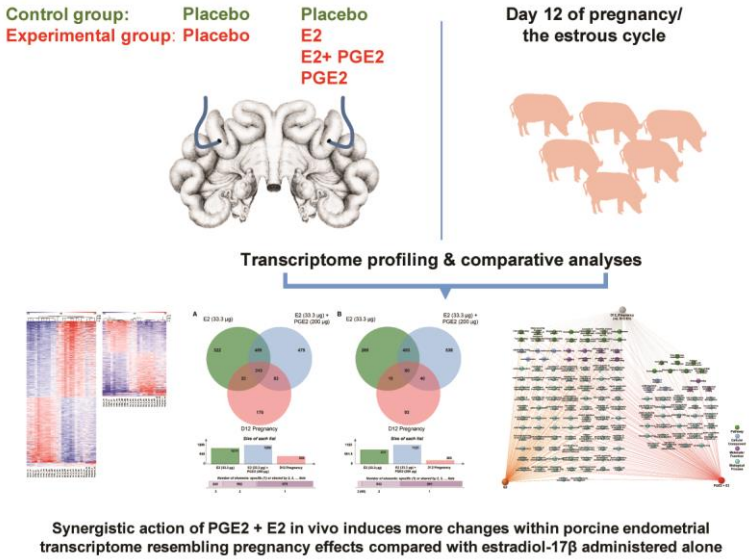


Figure 7. Expression of selected target genes determined by real-time RT-PCR in porcine endometrial samples treated in vivo with a placebo (control) or E2 (33.3 μg/infusion) or E2 (33.3 μg/infusion) together with PGE2 (200 μg/infusion) and on day 12 of the estrous cycle (D12 Cyclic) and pregnancy (D12 Pregnant). Data are presented as the mean ± SEM. The expression of genes was normalized against the geometrical mean of *RPL13A* and *GAPDH* expression values. Different letters indicate statistically significant differences (two-way ANOVA, followed by the Bonferroni post-test; $p<0.05$) in the local-indirect effects of E2+PGE2, E2 or PGE2 on gene expression. Asterisks (* $p<0.05$; ** $p<0.01$) indicate the significant differences within the direct local effects of E2+PGE2, E2, or PGE2 on gene expression. (A) caspase 3 (*CASP3*); (B) deleted in malignant brain tumors 1 (*DMBT1*); (C) growth differentiation factor 15 (*GDF15*); (D) kelch like family member 14 (*KLHL14*); (E) lysophosphatidic acid receptor 3 (*LPAR3*); (F) mucin 4, cell surface associated (*MUC4*); (G) prosaposin isoform A (*PSAP*); (H) serpin family B member 7 (*SERPINB7*); (I) solute carrier organic anion transporter family member 2A1 (*SLCO2A1*)); (J) secreted phosphoprotein 1 (*SPP1*); (K) transient receptor potential cation channel subfamily V member 6 (*TRPV6*); (L) Wnt family member 5A (*WNT5A*). Results of gene expression on day 12 of the estrous cycle and pregnancy presented on panels A-H and K-L have been published in [13].

FIGURE 7



Graphical Abstract



Synergistic action of PGE2 + E2 in vivo induces more changes within porcine endometrial transcriptome resembling pregnancy effects compared with estradiol-17β administered alone

Table 1. The selected results of DAVID functional annotation clustering for differentially expressed genes in the porcine endometrium in response to synergistic action of E2 and PGE2.

Selected functional terms of overrepresented annotation clusters	Annotation cluster enrichment score
UP REGULATED	
Extracellular exosome (405; 1.84) extracellular vesicle (405; 1.83)	37.20
Focal adhesion (80; 2.61)	14.58
Vasculature development (98; 2.26); blood vessel development (94; 2.30); angiogenesis (75; 2.56)	11.64
Cell migration (153; 1.82)	11.06
Glucose metabolic process (37; 2.73)	8.41
Regulation of signal transduction (265; 1.37)	8.23
Mitotic cell cycle (122; 1.78); cell cycle process (141; 1.46)	7.93
Regulation of protein phosphorylation (138; 1.50)	6.27
Antigen processing and presentation (38; 2.36)	5.24
Cadherin binding involved in cell-cell adhesion (45; 2.15)	5.09
Regulation of protein transport (86; 1.62)	5.02
Response to hypoxia (41; 2.07)	4.70
Tissue migration (38; 2.26); epithelial cell migration (35; 2.16)	4.67
Transforming growth factor beta receptor signalling pathway (28; 2.48)	4.45
Innate immune response-activating signal transduction (35; 2.03); activation of innate immune response (35; 1.97); positive regulation of innate immune response (39; 1.88)	3.79
Signal transduction by protein phosphorylation (92; 1.48); (74; 1.53)	3.72
Nuclear transport (50; 1.49)	2.49
In utero embryonic development (42; 1.79); embryo development ending in birth or egg hatching (56; 1.35); chordate embryonic development (55; 1.34)	2.29
Regulation of peptide transport (28; 1.89); peptide hormone secretion (29; 1.70); hormone transport (33; 1.52); hormone secretion (32; 1.53); signal release (38; 1.28)	2.12
Reproductive system development (46; 1.53); reproductive structure development (45; 1.52); developmental process involved in reproduction (56; 1.24)	1.93
DOWN REGULATED	
Transcription regulation (180; 1.45); positive regulation of RNA biosynthetic process (118, 1.51); regulation of gene expression (300; 1.20);	4.28
Cell morphogenesis (101; 1.42)	2.38
Endocrine and other factor-regulated calcium reabsorption (11; 4.85); Calcium Signalling pathway (19; 2.10)	2.28
Histone acetylation (18; 2.17)	2.09
Blood circulation (41; 1.42)	1.72
Positive regulation of ion transmembrane transporter activity (10; 2.53)	1.48
Steroid binding (12; 2.31); steroid hormone receptor activity (8; 2.33); steroid hormone mediated signalling pathway (16; 1.55); cellular response to steroid hormone stimulus (20; 1.45)	1.46
Calcium transport (12; 2.29)	1.45

BMP signalling pathway (15; 1.91); response to BMP (15; 1.78)	1.41

SUPPLEMENTAL FIGURES LEGENDS

Supplemental Figure 1. Gene expression distance matrix of analyzed samples generated in Geneplotter library performed for comparisons: PGE2 (200 µg/infusion) vs. placebo (A) and PGE2 (200 µg/infusion) + E2 (33.3 µg/infusion) vs. placebo. VSN-normalized microarray expression data was clustered based on pair wise correlation using all detectable probes. CTRL – endometrial samples collected from gilts assigned to the control groups (infusions of placebo to both uterine horns), EX_H – endometrial samples collected from gilts assigned to the experimental group (uterine horns receiving PGE2 (A) or PGE2+E2 infusions (B), EX_C – endometrial samples collected from gilts assigned to the experimental group (uterine horns receiving placebo infusion), cyclic – endometrial samples collected on day 12 of the estrous cycle, pregnant – endometrial samples collected on day 12 of pregnancy. In the control group, each horn received intrauterine placebo infusions. In experimental group, randomly selected horns within each gilt received hormonal infusions of PGE2 (200 µg/infusion) or PGE2 (200 µg/infusion) simultaneously with E2 (33.3 µg/infusion) whereas the contralateral horn received placebo infusions.